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Chemical Modification of DNA with Muta-Carcinogens. III. Reductive Alkylation of DNA with Mitomycin C.

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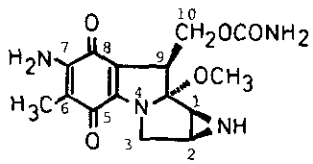
Mitomycin C (MMC) binds to DNA after its reductive activation by catalytic hydrogenation with Pd on charcoal. Three modified nucleotides, named MG-1, MG-2, and MA, were isolated from the modified DNA after enzymatic hydrolysis to 5'-nucleotides. The structures of these modified nucleotides were deduced from their ^{1}H-NMR and UV spectra, and from studies of the chemically transformed derivatives (hydrolysis, methylation, diazotization, and thioketonization). These three modified nucleotides were concluded to be 1,2-*trans*-2,7-diamino-1-(N^2-deoxyguanylyl)mitosene (MG-1), 2,7-diamino-1-(O^6-deoxyguanylyl)mitosene (MG-2) and 2,7-diamino-1-(N^6-deoxyadenylyl)mitosene (MA). The same modified nucleotides were identified in DNA extracted from the livers of rats treated with MMC.

Introduction

Mitomycin C (MMC) is a potent antibiotic and is also used clinically as an antitumor agent. Recently (*1*), a revised structure of MMC, the antipode of the structure reported previously, was proposed (I). It is well established that MMC requires reductive activation to become an alkylating agent of DNA (*2*). The action of MMC as a bioreductive alkylating agent of DNA is believed to be, at least in part, responsible for its effectiveness as an antitumor agent. Bifunctional alkylation of DNA leading to crosslinking of the two strands was considered to be the direct cause of the cytotoxicity of the drug (*3*), although the monofunctional attachment, which occurs 10 to 20 times more frequently than the crosslinking, has also been implicated as biologically significant damage to DNA (*2*). Elucidation of molecular aspects of this alkylation reaction of DNA by MMC should provide basic information for the development of more effective antitumor agents. Recently, Moore suggested that positions 1 and 10 of MMC are binding sites of MMC with DNA (*4*), though no unambiguous evidence for the chemical structure of the modified DNA has yet been obtained. Very recently, we reported (*5*) the alkylation of 5'-guanylic acid by reductively activated MMC and showed the structure of MMC-bound 5'-guanylic acid to be 1,2-*cis*-2,7-diamino-1-(5'-guanylyl)mitosene (Fig. 1). The other nucleic acid derivatives modified with MMC are uridyl derivatives (Fig. 1) reported by Tomasz and Lipman (*6*), but the products were obtained by reaction of MMC and uridylic acid derivatives under acidic conditions and without reductive activation of MMC.



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In this article, we review the isolation and structural identification of monofunctional alkylation products of DNA obtained with reductive activated MMC (*7,8*). DNA was modified chemically by catalytic reduction, as well as *in vivo* in male Wistar rats (*8*).

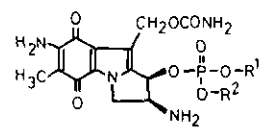

FIGURE 1. Structures of modified ribonucleotides: (1) R^1 = uridyl-5', R^2 = H; (2) $R^1 = R^2$ = uridyl-5'-; (3) R^1 = P(OH)OOP(OH)OO-(uridyl-5')-, R^2 = H; (4) R^1 = guanyl-5'-, R^2 = H.

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Structure of the Nucleotides Modified with MMC

MMC binds to DNA only after reductive activation under neutral conditions. We used catalytic hydrogenation with 5% Pd on charcoal and H_2 gas for reductive activation of MMC. About one molecule of MMC was bound per 200–300 nucleotides in DNA, as estimated from the UV spectrum of the modified DNA. Analysis of an enzymatic hydrolysate of the modified DNA showed the presence of three modified nucleotides, MG-1, MG-2, and MA. More than 80% of the binding of MMC to DNA could be accounted for by the formation of MG-1, MG-2, and MA.

The UV spectra of MG-1, MG-2, and MA have absorption maxima at ca. 310 nm, suggesting that these modified nucleotides contain a mitosene moiety, because the mitosene chromophore has an absorption maximum at 310 nm (*9*).

MG-1, MG-2, and MA were hydrolyzed completely by treatment with 1 N HCl. MG-1 gave guanine (45%) and xanthine (15%), and MG-2 gave guanine quantitatively, suggesting that MG-1 and MG-2 are deoxyguanylic acid–MMC adducts. MA gave adenine quantitatively, suggesting that MA is a deoxyadenylic acid–MMC adduct.

When these modified nucleotides were treated with alkaline phosphatase, less polar products (probably the corresponding nucleosides) were obtained quantitatively. This finding and the observation that these nucleotides were all resistant to phosphodiesterase suggest that these compounds have a monophosphate group at the 5′-position of 2′-deoxyribose.

^{1}H-NMR spectra were obtained in DMSO-d_6-CF_3COOD. The use of completely anhydrous solvent and exchange of hydrogen of NH and OH made it possible to assign all the signals. Assignment of proton signals was performed by homonuclear decoupling experiments and by comparing the spectra obtained with those of model compounds such as mitosenes and nucleotides. The results are shown in Figure 2.

The ^{1}H-NMR spectra and the results of hydrolysis of MG-1, MG-2, and MA suggest that the binding sites of nucleic acid base moieties are at heteroatoms of the purine rings. These modified nucleotides were hydrolyzed with 1 N HCl after methylation with trimethylsulfoxonium iodide in DMSO. Both MG-1 and MG-2 gave guanine (4–10%), 3-methylguanine (1%), 1-methylguanine (2–3%), and 7-methylguanine (4–5%). MG-1 also gave xanthine (4%). MA gave adenine (trace), 1-methyladenine (14%), 3-methyladenine (11%), and 7-methyladenine (0.5%). In all cases, some other products were observed, but not O^6-methylguanine from MG-2 and not N^6-methyl- or N^6,N^6-dimethyladenine from MA. These results suggest that the methylated positions (the 1-, 3-, and 7-positions) and the glycosylated position (the 9-position) of the purine rings are not the binding sites. Therefore, the binding site of MA is at the N^6 atom of adenine, and those of MG-1 and MG-2 are at the O^6 or

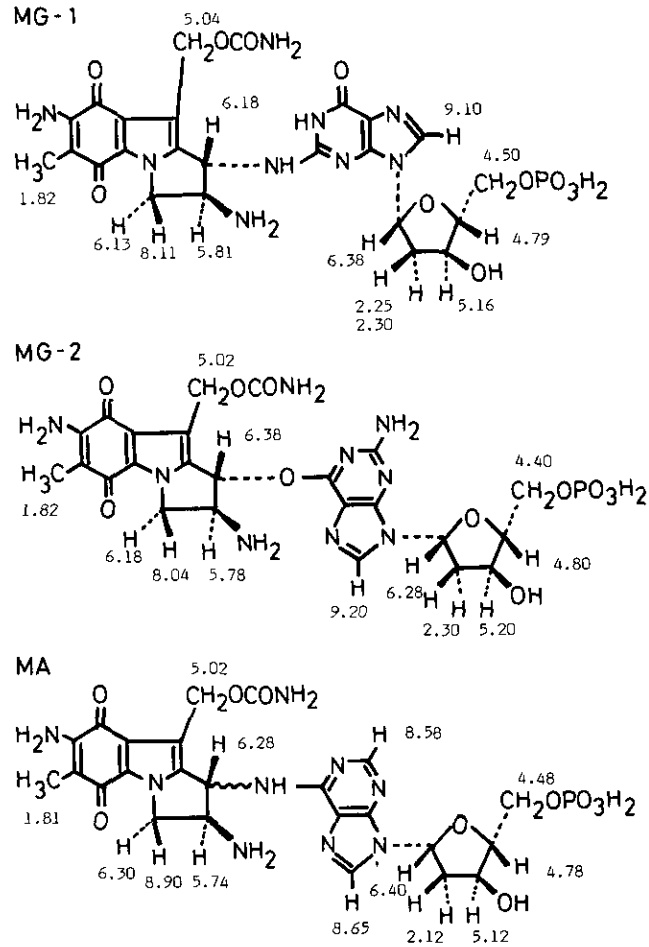

FIGURE 2. Structures of MA, MG-1, and MG-2 and their ^{1}H-NMR (δ from TMS in DMSO-d_6 containing 5% of CF_3COOD).

N^2 atom of guanine. For unambiguous determination of the binding sites of the guanine moieties of MG-1 and MG-2, the guanine moieties were chemically transformed. Acid hydrolysis of MG-1 and MG-2 after treatment with P_2S_5 gave 6-thioguanine from MG-1 (30%), but this was not obtained from MG-2. This suggests that the O^6 atom of the guanine moiety is blocked in MG-2 but not in MG-1. In addition, MG-1 and MG-2 were hydrolyzed with HCl after treatment with $NaNO_2$. The free amino group of guanine should be diazotized easily. Guanine (10%) together with xanthine (20%) was obtained from MG-1, but only xanthine (30%), not guanine, from MG-2. From these results, the binding sites of MG-1 and MG-2 were determined to be at the N^2 and O^6 atom, respectively. Therefore, the structures of MG-1, MG-2, and MA are as shown in Figure 2 (*7,8*). The structure of MG-2 was confirmed by Tomasz et al (*9*). They isolated MG-2 from the hydrolysate of the dinucleotide GpC modified with reductively activated MMC, and determined the configuration at positions 1 and 2 to be *trans*.

Modification of DNA *in Vivo*

DNA extracted from the liver of a rat treated with MMC was modified with MMC. About one molecule of MMC was bound per 1–2 × 10^4 nucleotides, as estimated from the UV spectrum of the modified DNA (ratio of absorbance at 260 nm to that at 310 nm). Enzymatic hydrolysis gave MG-1, MG-2, and MA in a ratio of 1:5:2 (Fig. 3) (*8*). The yields of these three modified nucleotides accounts for most of the MMC bound to DNA; the amounts of other nucleotides modified with MMC, if any, were very small. MMC bound preferentially to guanine in DNA, especially at the O^6 atom.

Reaction Mechanism

The mechanism of formation of these modified nucleotides was not established unequivocally, but in view of previous results, we propose that the mechanism of the reaction is as shown in Figure 4. MMC is reduced to the corresponding hydroquinone (MMC-H_2). This reduction enhances the electron-donating ability and aids elimination of the 9a-methoxy group. In the demethoxy product (demethoxy-MMC-H_2), cleavage of the aziridine ring is facilitated: position 1 is now benzylic and is activated by many electron-donating groups. The carbonium ion (MS-cation) formed by the opening of the

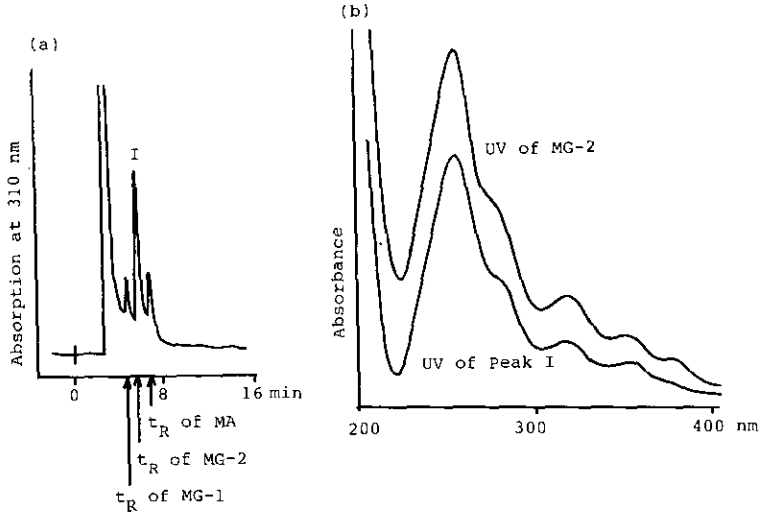

FIGURE 3. HPLC of hydrolysate of DNA extracted from the liver of a rats treated with MMC (*a*) and the UV spectra of peak I and MG-2 (*b*). Column: Polygosil $_5C_{18}$, 4.6 mm ID × 25 mm; solvent: CH_3CN(10%)–0.3%NH_4Cl aq.(90%); flow rate: 0.8 mL/min.

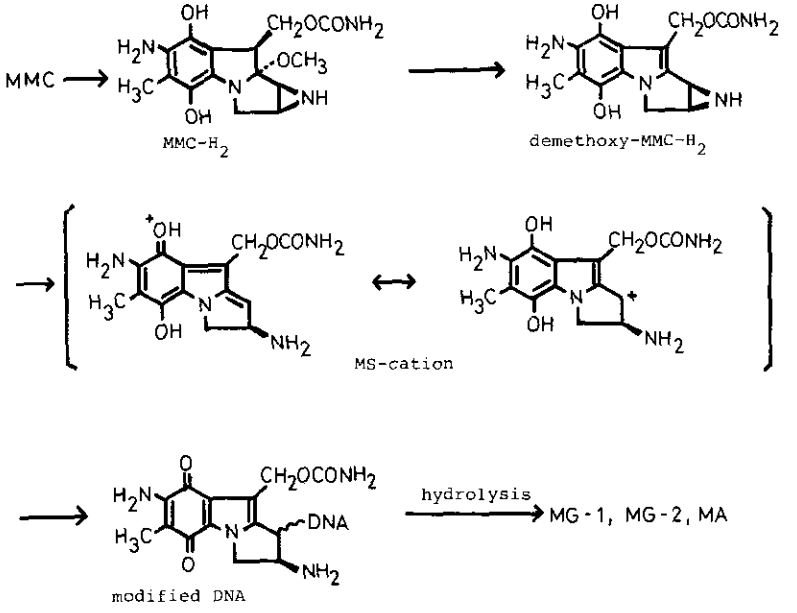

FIGURE 4. Reaction mechanism of modification of nucleic acids by reductively activated MMC.

aziridine ring has a planar structure which can intercalate into DNA and can thus approach position 2 or 6 of the purine bases. Covalent binding then occurs between position 1 of mitosene and heteroatom at position 2 or 6 of the purine bases. The products might be oxidized by excess MMC or MMC derivatives (or by other cellular components *in vivo* and *in vitro*) or during the work-up procedures to give MG-1, MG-2, and MA.

Conclusion

The structures of the nucleotides modified with reductively activated MMC were determined to be as shown in Figure 2. The reaction established chemically occurs both *in vitro* and *in vivo*. The present findings represent a first step in understanding the molecular basis of the action of MMC as a bioreductive alkylating agent. The reductive activation and the mode of modification of nucleotides in DNA must, at least in part, play a role in the carcinostatic or carcinogenic mechanisms of MMC. These findings may contribute to the development of more effective antitumor-active MMC derivatives.

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